Composition and Dynamics of Protein Complexes Measured by Quantitative Mass Spectrometry of Affinity-Purified Samples

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Summary

Multiple protein complexes are fundamental parts of living systems. Identification of the components of these complexes and characterization of the molecular mechanisms that allow their formation, function and regulation can be done by affinity purification of proteins and associated factors followed by mass spectrometry of peptides. Speed and specificity for the isolation of complexes from whole cell extracts improved over time together with the reliable identification and quantification of proteins by mass spectrometry. Relative quantification of proteins in such samples can now be done to characterize even relatively non-abundant complexes. We describe here our experience with proteins fused with the Z domain, derived from staphylococcal protein A, and IgG affinity purification for the analysis of protein complexes involved in RNA metabolism in the budding yeast *Saccharomyces cerevisiae*. We illustrate the use of enrichment calculations for proteins in purified samples as a way to robust identification of protein partners. While the protocols presented here are specific for yeast, their principles can be applied to the study of protein complexes in any other organism.

Key words: Mass spectrometry; Affinity purification; Protein complexes; Complexes dynamics.

1 Introduction

Affinity purification of protein complexes originated in immunoprecipitation assays using antibodies against one of the complex components. Generic strategies for protein complexes characterization became available through the development of tags which, when fused with proteins of interest, are used for affinity purification. One of the most successful affinity isolation strategy involved the use of two purification steps in the Tandem Affinity Purification method [1]. The isolated complexes can be used for functional and structural studies [examples for pre-ribosomal intermediates reviewed in 2]. To identify the components of the purified samples, they are subjected to trypsin (Lys-C or other protease) digestion, followed by the analysis of the obtained peptide mix through liquid chromatography coupled with high resolution mass spectrometry. Technical developments steadily increased the number of proteins identified from purified samples or whole cell extracts and also increased the peptide coverage of the detected proteins. Improvements in data analysis strategies allowed the combination of peptide signals for a given protein in a sample and comparisons with peptide signals for the same protein in other purified complexes or under modified conditions.

The introduction of superparamagnetic beads coated with affinity reagents led to the improvement in the recovery of very large complexes, such as polysomes [3] and allowed shorter incubation times [4]. The original TAP tag contains two 58 amino acid long Z domains, engineered based on staphylococcal protein A structure when bound to the Fc region of human IgG1 [5]. Using beads covalently bound to IgGs leads to much lower background compared with Sepharose or agarose-based IgG formulations. Thus, for most applications, we only use a single step purification, based on the binding of the two Z domains of a fusion protein of interest to rabbit IgG on superparamagnetic beads.

Mass spectrometry fundamental improvements, such as the development of the Orbitrap technology [6], together with better separation systems for peptide separation at very low flows, allow the identification and quantification of thousands to dozens of thousands of peptides in a single run [7]. Finally, crucial to the analysis of results is the ability to compare many independent runs and combine the results for visualization and further exploration. We found, for example, Perseus [8] to be efficient for manipulation of mass spectrometry data and interactive visualization of the results, including their annotations. Other options for the analysis of MaxQuant [9] results, based on the R language and libraries are Proteus [10] and the Bioconductor packages artMS [11] or DEP [12]. As soon as a corpus of mass spectrometry results is generated, it can be useful to present these data in an web-accessible interactive interface. For example, we included our published results on yeast nonsense-mediated

mRNA decay (NMD) and NMD-associated complexes [13] in a web interface for rapid and interactive visualization of the results: hub05.hosting.pasteur.fr/NMD_complexes/. This interface presents both comparisons of intensity of signal for a given protein in two conditions, but also a derived measure that takes into account the relative abundance of proteins and adjust the intensity values accordingly. The "enrichment" value calculated this way allows a better discrimination of specific from non-specific associations (for an example, see **Fig. 1a**)

Even small protocol changes can have a dramatic effect on the result of an affinity purification experiment. For example, we had several failed experiments due to decreased specific activity of the protease used to elute complexes under mild conditions. Thus, we favor more robust elution protocols. High concentrations of urea or guanidine were relatively inefficient in eluting complexes. However, high salt treatment using 2 to 4 M $MgCl_2$ or denaturing agents, such as SDS coupled with heat were effective. These methods are both safer and cheaper than using a specific protease, but cannot be used to elute complexes for further purifications or biochemical tests. Elution conditions will affect subsequent steps of protein digestion and liquid chromatography. To remove excess SDS from eluates, for example, one can use a methanol-chloroform precipitation, which strongly reduces the amount of detergent in the sample [14].

Affinity purification of complexes is especially informative when performed under a variety of conditions that test the constitution or dynamics of the studied multiprotein assembly. For example, the importance of RNA to an RNA-protein complex can be tested by adding an RNase, such as the micrococcal nuclease [e.g. 15], during one of the incubation steps. Complex formation can be dissected by performing the purification in strains or cell lines in which one of the complex components is absent. Finally, the relative importance of protein domains for interactions can be deduced from purifications performed with truncated proteins, as illustrated in **Fig. 1b**.

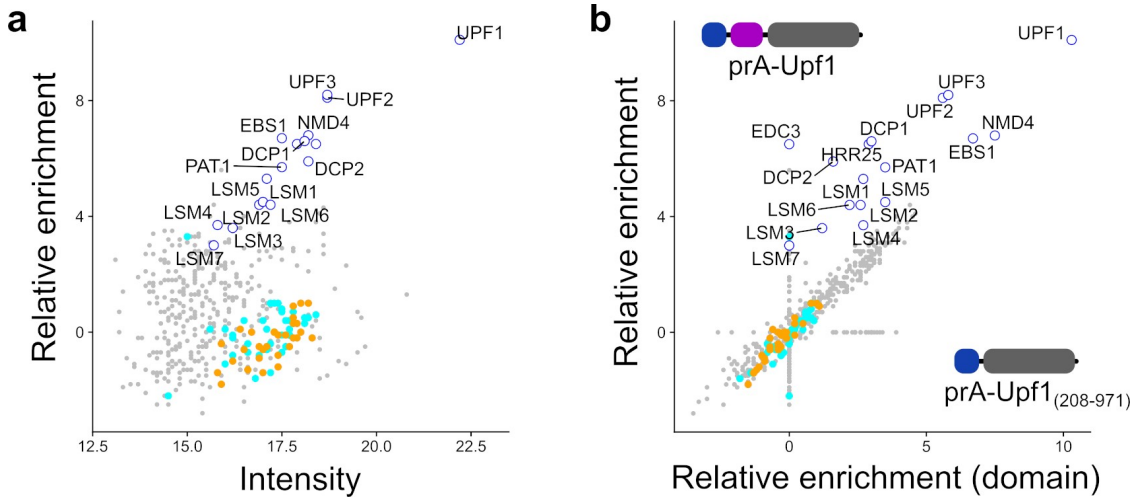

Figure 1. Examples of data visualization for quantitative comparisons of proteins in

purified samples. Data are from [13]. **a)** Relative enrichment in the purified sample, vertical axis, corrects the obtained intensity values, horizontal axes, based on the abundance of each protein in a total extract. Labeled proteins are components of NMD complexes. Ribosomal proteins of the large subunit (cyan) and of the small subunit (orange) are also shown.
b) Comparison of enrichment to assess the involvement of a protein domain in the association of partner proteins in NMD complexes. Two variants of tagged Upf1 were used, one lacking the cysteine, histidine rich (CH) N-terminal domain, for purification. The tag used for the purification is depicted in blue, with the CH domain in purple. The values on the axes were log2 transformed (one unit difference corresponds to a factor of 2). Proteins showing a displacement of signal towards the upper left quadrant are likely to depend on the missing domain for their association with the RNA helicase Upf1.

The following sections provide detailed protocols for the purification of proteins fused with the original TAP tag [1], although a single purification step is used. They include the preparation of IgG-coupled magnetic beads, yeast cells lysis, the purification procedure, elution and eluate treatment protocols. Digestion of the proteins to peptides and an example of LC-MS/MS protocol is also provided.

2 Materials

2.1 Yeast growth medium

1. YPD medium: 2% D-glucose, 1% Difco yeast extract (BD, 212720), 1% Difco Bactopeptone (BD, 211677). Autoclave at 110°C for 20 minutes.

2.2 Magnetic beads preparation

1. Dynabeads M-270 epoxy (Dynal).

2. Rabbit IgGs (Sigma I5006-100MG).

3. 0.1 M sodium phosphate pH 7.4 (for 100 ml of 1M stock: 2.62 g $NaH_2PO4^* H_2O$ and 14.42 g $Na_2HPO_4 * 2 H_2O$).

4. Ammonium sulfate solution (19.82 g of $(NH_4)_2SO_4$ in 50 ml 0.1 M sodium phosphate buffer, pH 7.4).

5. Glycine-HCl (3.75 g glycine, 4.3 ml of 37% HCl, for 500 ml).

6. PBS – phosphate buffered saline (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM)

7. Triethanolamine (0.1M, pH 8.8).

2.3 Cell lysis and purification

1. Lysis buffer: 20 mM HEPES K, 100 mM potassium acetate, 5 mM $MgCl_2$, protease

inhibitor (Roche complete EDTA-free, ref 11 873 58001), pH 7.4.

2. Wash buffer: 20 mM HEPES K, 100 mM potassium acetate, 5 mM $MgCl_2$, 0.5% Triton X-100, pH 7.4.

3. Acid-washed glass beads, 425-600 µm (e.g. Sigma G8772).

4. SDS elution buffer: Tris HCl 10 mM, EDTA 1 mM, 2% SDS, pH 8.

5. Methanol (mass-spectrometry grade).

6. Chloroform (mass-spectrometry grade).

7. FastPrep-24 (MPBio) or equivalent bead beater vortex with adapter for 50 ml tubes.

8. Magnetic stand for 1.5 ml Eppendorf tubes (e.g. MagRack 6, Cytiva).

2.4 Chromatography and mass spectrometry

1. Tris HCl 0.1M, pH 8, with 8 M urea.

2. TCEP (tris(2-carboxyethyl)phosphine) 0.5 M : Dilute 1/5 in Tris 0.1 M, Urea 8M, pH 8 as working solution.

3. Iodoacetamide (IAA) or chloroacetamide (ClAA): 0.5 M solution (to be prepared freshly in Tris HCl 0.1 M, urea 8M, pH 8).

4. CaCl2 0.5 M stock Solution in 0.1 M Tris HCl pH 8.

5. Endoproteinase Lys-C (20ug, WAKO SOBIODA Ref. 125-05061): 20 µg are diluted by 100 µl of Tris 0.1 M Urea 8 M pH8 (0.2 µg/µl). 20µl aliquots to be stored at -80°C.

6. Trypsin Gold MS Grade Promega V5280 (100µg) 100 µg are diluted in 500 µl 0.1 M TrisHCl pH 8 (0.2 ug/µl). 20µl aliquots to be stored at -80°C.

7. 0.1% trifluoroacetic acid (TFA).

8. Chromatography solvent formulation A: 0.1% formic acid/ 99.9% water (v/v).

9. Chromatography solvent formulation B: 0.1% formic acid/ 80% acetonitrile/19.9% water (v/v/v).

10. SpeedVac, Thermomixer or equivalent devices.

11. Omics C18 100 µl tips (Agilent).

12. EASY-Spray column (ThermoFisher Scientific, C18, particle size 2 µm, length 150mm, pore size 100°A, diameter 75 µm), or equivalent.

13. EASY-nLC 1000 chromatogrphy column (ThermoFisher scientific), or equivalent.

3 Methods

3.1 Preparation of IgG coupled superparamagnetic beads

1. Wash 300 mg beads (about $2x10^{10}$ beads) with 20 ml of 0.1M sodium phosphate buffer (pH 7.4). Vortex 30 seconds and incubate 10 minutes at room temperature. Recover the beads with a magnetic support (about 2 minutes). Repeat the was. Suspend the beads in 4.5 ml sodium phosphate buffer (pH 7.4).

2. Thaw 1.5 ml rabbit IgGs[NOTE1] stock solution (10 mg/ml in PBS) and add it to the 4.5 ml of magnetic beads suspension. Slowly add 3 ml of 3 M ammonium sulfate solution. Incubate on a spinning wheel at 30°C for 20 hours.

3. The next day, recover the beads on a magnetic support. Wash with 7.5 ml PBS, followed by 1.5 ml glycine-HCl 0.1M, pH 2.5 buffer.

4. Wash sequentially with 7.5 ml each of the following solutions: PBS; Triethanolamine 0.1 M, pH 8.8; PBS; PBS with 0.5% Triton X100; PBS.

5. Store the IgG-beads preparation as a suspension in 1.5 ml PBS containing 0.01% Thimerosal at 4°C. This corresponds to an approximate concentration of beads of 0.2 mg/ml. This preparation should be sufficient for about 70 purifications[NOTE2].

3.2 Preparation of a total yeast extract

1. Prepare an initial culture of yeast expressing the protein of interest in fusion with the TAP tag by seeding the appropriate strain in the evening in 200 ml of YPD medium. In the next morning, dilute the saturated culture, grown at 30°C with agitation, to an approximate optical density of 0.4 in 2 to 4 liters of YPD. Allow to grow for 4 to 6 hours until the optical density reaches an approximate value of 2.

2. Recover cells by centrifugation for 10 minutes at 3500 rpm in a large volume centrifuge. Wash the cells with 30 ml cold water and transfer to a 50 ml tube. Centrifuge again for 10 minutes at 3500 rpm, at 4°C, and remove the supernatant. Cells can be stored at -80°C until lysis or used directly. Typical yields are 3 to 5 gm cells.

3. Suspend cells in an equal volume of lysis buffer by thorough pipetting. Transfer to a Falcon tube containing 20 ml of acid-washed glass beads. Perform a series of three 40 seconds bead beating in the FastPrep (6 m/s), with chilling intervals of 2 minutes in between.

4. Recover the cell lysate by centrifugation for 20 minutes at 3500 rpm. Its appearance should be pale yellow. Keep a small sample of total extract at -20°C, to be able to control the presence of the tagged protein and the total protein concentration.

3.3 Purification of tagged proteins

1. Prepare IgG-magnetic beads for purification by washing, for each purification, 20 µl of stock beads suspension, with 200 µl lysis buffer. Resuspend in lysis buffer.
2. Mix the total cell lysate that contains a TAP-tagged protein with the IgG-magnetic beads suspension. Allow binding to proceed between 20 and 120 minutes on a rotating wheel at 4°C. Preliminary tests might be required to establish the optimal incubation period.
3. Remove the magnetic beads from solution using a magnetic stand. Suspend the beads in 1 ml lysis buffer and transfer to 1.5 ml Eppendorf tubes. Remove the supernatant.
4. Wash the beads five times with 1 ml each of wash buffer. Each time, resuspend the beads in the buffer.
5. Wash again the beads with 1 ml buffer without detergent. Add 50 µl SDS elution buffer[NOTE3] and resuspend the beads. Incubate 10 minutes at 65°C. Carefully recover the eluate and proceed to performing controls of purification efficiency by denaturing polyacrylamide gels, immunoblots or follow up with precipitation for mass spectrometry analysis.

3.4 Protein precipitation with methanol-chloroform[NOTE4]

1. Adjust the eluate volume to 100 µl with water. Add 400 µl high purity methanol and vortex throughly.
2. Add 100 µl chloroform and vortex.
3. Add 300 µl water. Mix thorougly for 1 minute. Mixing well at this step is important for phase separation. The solution becomes turbid.
4. Centrifuge 30 minutes at 14 000 rpm at 4°C. For eluates rich in protein a white precipitate ring can be observed at the interface of the two phases.
5. Remove upper phase (methanol and water) and leave in the tube the lower phase (chloroform). Pay attention to leave a small amount of the upper phase and not to remove the precipitate.
6. Add 300 µl methanol and mix thorougly. Leave 10 minutes at -20°C. Centrifuge for 20 minutes at 14 000 rpm and 4°C. Remove the supernatant. Sometimes, e white precipitate can be observed at the bottom of the tube or on the tube wall.
7. Leave for about 5 minutes at room temperature to allow evaporation of trace amounts of chloroform and methanol. Store at -20°C until processing for mass spectrometry.

3.5 Proteolytic lysis of proteins for mass-spectrometry

a) Reduction and alkylation

1. Dilute each sample in 30 µl of 0.1 M Tris HCl, pH 8, 8 M Urea. Vortex.
2. Add 1.5 µl TCEP 0.1 M and leave at room temperature (20-22 °C) for 30 min.
3. Add 0.6 µl of freshly prepared IAA (or ClAA) 0.5M. Leave at room temperature for 30 min in the dark.

b) Protein digestion

4. For each sample, add the endoproteinase Lys-C (5 µl) at 0.2 µg/µl in 0.1M Tris HCl, 8 M urea. Incubate at 37°C for at least 6 hours.
5. Add to each sample 94 µl 100 mM Tris HCl, pH 8, 0.5 µl of $CaCl_2$ (0.5 M stock solution) and 2.5 µl of stock trypsin at 0.2 µg/µl. Incubate at 37°C under agitation overnight, with a Thermomixer device.
6. Repeat the addition of $CaCl_2$ and trypsin and incubate at 37°C with stirring, using a Thermomixer, for 5 hours.
7. Add 6 µl of formic acid for a final concentration of 5%.

c) Peptides desalting

8. Desalting uses OMIX C18 100 µl tips. Perform 5 washes of the OMIX support with pure acetonitrile followed by 5 washes of the OMIX support with 0.1% TFA solution.
9. Load each sample by performing 20 back and forth pipetting through the tips.
10. Perform 7 washes of each sample with 150 µl 0.1% TFA solution, each.
11. To elute peptides perform 20 back and forth pipetting in each tip with 150 µl of a mix in equal volumes of acetonitrile and 0.1% TFA.
12. Finally, rinse the tip with 150 µl of a 3/1 volume mix of acetonitrile and 0.1% TFA solution. Further processing requires dried material, that can be obtained with a SpeedVac. Store the eluted peptides at -20°C.

3.6 Liquid chromatography coupled with mass spectrometry

1. Add 12 µl LC-MS buffer: water/acetonitrile/TFA (96/4/0.1, volume to volume mix). Mix well. Centrifuge at maximum speed for 10 min at 4°C. Transfer 10 µl to the LC-MS injection vial. Store at 4° C until the start of the analysis.
2. Liquid chromatography analysis is performed with a specific capillary column, such as the EASY-nLC 1000. For each sample, 3 µl of peptide mixtures are loaded on an EASY-Spray column. Use a column temperature of 50°C at a flow rate of 250 nl/min.

3. A linear gradient over 120 minutes is used for gradual peptide elution during each run. An example of how the gradient can be set up is the following:

Time (minutes)	Duration (minutes)	Mixture [%B]
0	0	4
70	70	25
95	25	45
100	5	95
105	5	95
105	0	4
120	15	4

4. For LTQ-Orbitrap Velos acquisition full MS scans are acquired in the Orbitrap mass analyzer over the m/z 300–1700 range with resolution 60,000 (m/z 400). The target value is $5x10^5$. The LTQ Orbitrap is operated in a data-dependent mode, switching automatically between one full-scan and subsequent MS/MS scans of the 20 most abundant peaks (Top20 method by CID). Ions with charge state 2 are selected for sequencing and fragmented in the ion trap with normalized collision energy of 35%, activation Q = 0.25, activation time of 10 msec, and one microscan. The target value is 10^4. The ion selection threshold is 500 counts. Dynamic exclusion is enabled; with an exclusion list size of 500 and an exclusion duration of 30 s. Standard MS parameters are set as follows: 1.9 kV spray voltage and 275 °C heated capillary temperature.

3.7 Identification of proteins and raw quantitation of results

1. Processing of RAW output from the mass spectrometer can be done with MaxQuant with default parameters unless otherwise specified (http://www.maxquant.org). Database searches are performed with the built-in *Andromeda* search engine against a reference proteome (in our case the *S. cerevisiae* one recovered from Uniprot). MaxQuant provides a “contaminants.fasta” file including common laboratory contaminant proteins that is automatically added to the list of proteins for the *in silico* digestion.

2. Precursor mass tolerance is set to 6 ppm in the main search, and fragment mass tolerance is set to 0.5 Da (these parameters depend on the specific mass spectrometer). Digestion enzyme specificity is set to trypsin with a maximum of two missed cleavages. A minimum peptide length of 7 residues is required for identification. Up to five modifications per peptide are allowed; acetylation (protein N-terminal), and oxidation (Met) are set as variable modifications. Carbamidomethylation (Cys) is set as a fixed modification. Peptide and protein false discovery rates (FDR) are both set to 1%.*

3. Relative, label-free quantification of proteins can be done using the MaxLFQ

algorithm integrated into MaxQuant. Typical parameters are as follows: Minimum ratio count set to 2, and the FastLFQ option enabled. Proteins that shared same identified peptides are combined into a single protein group[NOTE5].

3.8 Interpretation of quantitative MS results

1. To identify proteins that interact with a tagged protein of interest in the presence of a vast number of background binding proteins, replicates of affinity-enriched bait samples are compared to a set of negative control samples. The tools used to perform these comparisons range from spreadsheet calculations to R packages or specific visualization and data analysis programs, such as the Perseus environment. Here, we describe a sequence of operations, using Perseus, for the analysis of MaxQuant output.

2. The “proteingroups.txt” file from MaxQuant is loaded and then entries in the for “Reverse”, “Only identified by site modification”, and ‘Potential contaminants” are removed before further processing. Protein groups identified by a single “razor and unique peptide” are removed from the data set.

3. Protein group LFQ values are log2 transformed. Missing LFQ values are assumed to represent mostly low abundance proteins that are below the MS detection limit. Imputation of these missing values is performed separately for each sample and missing values are replaced with random low level values. Alternatively, imputation can be done by using the a constant value equal to the lowest LFQ value for each run.

4. In the presence of at least three replicate experiments, differences in relative abundance of proteins in different conditions are evaluated using a t-test. T-test results can be visualized with volcano plots, in which, the difference between two samples is represented on the horizontal axis and the p-value of the t-test for difference for each protein between sample and control is represented on an inverse log scale. Enriched interactors appear in the upper right section of the plot[NOTE6].

4 Notes

1. The specific use of rabbit IgG is important here for proteins fused with the Z domain of protein A, which is present in the original TAP tag. Binding of this domain to the Fc region of antibodies is type and species specific. Thus, human IgG1, IgG2 and IgG4 bind protein A, while goat IgGs, for example, have low affinity for protein A.

2. Beads can also be produced in the laboratory, by using mixtures of $FeCl_2$/$FeCl_3$ and silica coating and chemical modification, for price reduction and customization [16].

3. Elution can also be done in the presence of high salt concentrations, for example

with 200 µl of 2M or 4M $MgCl_2$. For specific purification types, such as the streptavidin-biotin purification, elution might not be necessary if streptavidin was rendered resistant to trypsin and Lys-C protease digestion [17].

4. Methanol-chloroform precipitation was developed to allow extraction of proteins and their separation from lipids and detergent [18]. It is also effective in the presence of high salt concentrations, such as 2 or 4 M $MgCl_2$. However, precipitation methods can lead to irreversible protein aggregation or protein loss. Such events can be avoided by using other preparation methods for samples that contain SDS, such as eFASP [19], one of the variants of the filter aided sample preparation protocol.

5. MaxLFQ is only one of several scores that have been proposed and used to compare relative amounts of the same protein in different samples. We obtained similar results using a variant of the Top3 method [20], in which only the three most intense peptides are used to compare protein amounts. In light of the continuous improvement of coverage and sensitivity of mass spectrometers, it is likely that the label-free quantification scores will also further evolve.

6. While volcano plots are frequently used for the visualization of quantification results based on mass spectrometry results, one should be aware that p-values themselves are random variables with a variance that can be important, especially if only three replicates have been performed. While a low variability of quantitative values obtained in several replicates for a given protein correlate with low p values (high on the vertical axis of usual volcano plot), the difference between sample and control should be the most important information extracted from a volcano plot. As stated in [21] "*no p-value can reveal the plausibility, presence, truth, or importance of an association or effect*". This analysis of the use of p-values also suggest that the term "statistically significant" should be banned from scientific communication.

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